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Evolutionary Dynamics of the Genomic Region Around the Blast Resistance Gene *Pi-ta* in AA Genome Oryza Species

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ABSTRACT

The race-specific resistance gene *Pi-ta* has been effectively used to control blast disease, one of the most destructive plant diseases worldwide. A single amino acid change at the 918 position of the Pi-ta protein was known to determine resistance specificity. To understand the evolutionary dynamics present, we examined sequences of the *Pi-ta* locus and its flanking regions in 159 accessions composed of seven AA genome Oryza species: *O. sativa*, *O. rufipogon*, *O. nivara*, *O. meridionalis*, *O. glaberrima*, *O. barthii*, and *O. glumaepatula*. A 3364-bp fragment encoding a predicted transposon was found in the proximity of the *Pi-ta* promoter region associated with the resistance phenotype. Haplotype network analysis with 33 newly identified *Pi-ta* haplotypes and 18 newly identified Pi-ta protein variants demonstrated the evolutionary relationships of *Pi-ta* haplotypes between *O. sativa* and *O. rufipogon*. In *O. rufipogon*, the recent directional selection was found in the *Pi-ta* region, while significant deviation from neutral evolution was not found in all *O. sativa* groups. Results of sequence variation in flanking regions around *Pi-ta* in *O. sativa* suggest that the size of the resistant *Pi-ta* introgressed block was at least 5.4 Mb in all elite resistant cultivars but not in the cultivars without *Pi-ta*. These findings demonstrate that the *Pi-ta* region with transposon and additional plant modifiers has evolved under an extensive selection pressure during crop breeding.

PLANT resistance (*R*) genes have evolved to fight against a wide range of pathogens in a race-specific manner where a particular *R* gene in a plant recognizes the corresponding avirulence (*AVR*) gene in a pathogen race (Flor 1971). Thus far, a number of *R* genes have been identified and characterized from diverse plant species. Most characterized *R* genes to date encode putative proteins with nucleotide binding sites (NBS) and leucine-rich repeats (LRR) (Hulbert *et al.* 2001). Most *R* genes are highly polymorphic and diversified, which is consistent with the ability to interact with diverse random molecules encoded by diverse pathogen *AVR* genes (Meyers *et al.* 2003; Bakker *et al.* 2006; Shen *et al.* 2006).

Blast disease, caused by the filamentous ascomycete *Magnaporthe oryzae* B.C. Couch [formerly *M. grisea* (T. T. Hebert) M. E. Barr] (Rossman *et al.* 1990; Couch and Kohn 2002), has been one of the major constraints to stable crop production. Currently, *Oryza sativa* and *M. oryzae* have been an excellent model pathosystem for uncovering the molecular coevolution mechanisms of host–pathogen (Valent *et al.* 1991; Talbot 2003). At least 80 race-specific *R* genes that confer resistance to specific pathogen races have been described in rice germplasm (Ballini *et al.* 2008). Eleven blast *R* genes (*Pi-ta*, *Pib*, *Pi2/Piz-t*, *Pi5*, *Pi9*, *Pi21*, *Pi36*, *Pi37*, *Pi-d2*, *Pikm*, and *Pit*) have been cloned, and most of them, except *Pi21* and *Pi-d2*, were also predicted to encode receptor proteins with NBS (Chen *et al.* 2006; Fukuoka *et al.* 2009; Jia *et al.* 2009b). In most cases, blast *R* genes are members of small gene families with a single family member required for resistance. *Pikm* and *Pi5* are exceptions that require two members of the same gene family for *Pikm*- and *Pi5*-mediated resistance, respectively (Ashikawa *et al.* 2008; Lee *et al.* 2009). Recently, a retrotransposon was predicted to be involved in the *Pit* resistance (Hayashi and Yoshida 2009).

The evolutionary dynamics and mechanisms of resistance mediated by *Pi-ta* is one of the best-studied *R*-genes. *Pi-ta* has been effectively deployed in the United States and around the globe for controlling blast disease (Bryan *et al.* 2000; Jia *et al.* 2000; Jia 2003; Jia *et al.* 2004a,b; Huang *et al.* 2008; Jia and Martin 2008; Wang *et al.* 2008; Jia *et al.* 2009a). *Pi-ta* encodes a predicted cytoplasmic protein with a centrally located NBS and

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a highly interrupted LRR domain (referred to as the LRD) at the carboxyl terminus that recognizes the corresponding avirulence gene *AVR-Pita*, triggering race-specific resistance. A single amino acid substitution, serine (Ser) to alanine (Ala) at the position of 918, in the LRD of the Pi-ta protein was demonstrated to determine the direct interaction with AVR-Pita and the resistance specificity to blast pathogen *M. oryzae* (Bryan *et al.* 2000; Jia *et al.* 2000). The resistant *Pi-ta* allele (Ala-918) was found in *O. sativa* and its ancestor *O. rufipogon* (Jia *et al.* 2004b; Huang *et al.* 2008). Surveys of *Pi-ta* nucleotide sequences with limited accessions of Oryza species have revealed that the degree of nucleotide diversity is higher at the intron of the *Pi-ta* gene (Jia *et al.* 2003; Huang *et al.* 2008; Wang *et al.* 2008; Yoshida and Miyashita 2009). Huang *et al.* (2008) further suggested that a selective sweep occurred recently at the *Pi-ta* gene in *O. rufipogon*, but the extent of selection around the *Pi-ta* genomic region has not been demonstrated in either *O. rufipogon* or *O. sativa*.

Knowledge of the historical introduction of the *Pi-ta* gene can help to understand the extent of selection at the *Pi-ta* locus. The landraces Tadukan and Tetep, containing *Pi-ta* and other blast *R* genes in chromosome 12, have been used as breeding parents for preventing blast disease worldwide. Tadukan was confirmed to be the *Pi-ta* donor for various Asian *japonica* cultivars (Rybka *et al.* 1997) whereas Tetep was the *Pi-ta* donor for the U. S. cultivars (Gravois *et al.* 1995; Moldenhauer *et al.* 1998; McClung *et al.* 1999; Gibbons *et al.* 2006; Moldenhauer *et al.* 2007). Recently, the large introgressed chromosomal segments surrounding the *Pi-ta* locus were identified in backcross BC_5 progenies and elite rice cultivars (Jia 2009). This suggests that the broad spectrum of the *Pi-ta* resistance in the United States may include the effects of other loci in the *Pi-ta* region, inherited as a "superlocus." Toward this end, *Ptr(t)*, a nuclear gene that is required for the *Pi-ta*-mediated resistance, was also mapped at the *Pi-ta* region (Jia and Martin 2008). Further determination of DNA sequences around the *Pi-ta* gene should help to determine the minimal genomic region that is essential for *Pi-ta*-mediated resistance.

The two cultivated rice species, *O. sativa* and *O. glaberrima*, belong to the AA genome of Oryza species. *O. rufipogon* and *O. nivara* are wild progenitors of the Asian rice *O. sativa*, whereas *O. barthii* is a wild progenitor of the African cultivated rice *O. glaberrima* (Linares 2002; Yamanaka *et al.* 2003; Londo *et al.* 2006). The comparison of *R*-gene diversity between cultivated rice and its wild ancestors is important to understand the selection effects of crop domestication and breeding.

The objectives of this study were (1) to characterize distributions of the *Pi-ta* allele in *O. sativa* and to detect the potential presence/absence of polymorphism(s) associated with the resistance phenotype; (2) to examine the molecular evolution and patterns of selection in the *Pi-ta* gene in *O. sativa* and *O. rufipogon*; (3) to analyze molecular diversity around the *Pi-ta* locus in AA genome Oryza species; and (4) to understand the pattern and extent of selection for *Pi-ta*-mediated resistance in Oryza species during crop domestication.

MATERIALS AND METHODS

Plant materials and DNA preparation: A total of 159 geographically diverse accessions of *O. sativa*, *O. rufipogon*, and five other closely related AA genome Oryza species were selected for this study. These included 43 Asian landraces, 18 U. S. domesticated cultivars, and 58 U. S.weedy rice strains in *O. sativa*; 28 geographically diverse accessions of *O. rufipogon*; 4 accessions of *O. glaberrima*; and 2 accessions each of *O. nivara*, *O. barthii*, *O. meridionalis*, and *O. glumaepatula* (Table S1). U. S. cultivars and weedy rice seeds were obtained from the USDA-ARS Dale Bumpers National Rice Research Center, and all Asian landrace accessions consisting of 15 *indica*, 7 *aus*, 3 *aromatic*, 12 *tropical japonica*, and 4 *temperate japonica* were obtained from Susan McCouch at Cornell University and the International Rice Research Institute. Plants were grown in greenhouses at Washington University and the University of Massachusetts. DNA extracted from 2- to 4-week-old seedlings was diluted to 2 ng/μl for further analysis.

Primer design and DNA sequencing: Primer pairs were designed using the Primer3 program (Rozen and Skaletsky 2000) to amplify overlapping fragments (~700 bp each) for *Pi-ta*, including 5′ upstream, 3′ downstream, and a coding region with an intron (Table S2). All primers were verified by BLAST against both 93-11 (*indica*) and Nipponbare (*japonica*) genome sequences. Primers were also designed to amplify 400- to 700-bp fragments of six flanking genes in the regions from 9.6 to 11.6 Mb on chromosome 12. The six flanking loci around the *Pi-ta* gene were LOC_OS12G16690 (9.6 Mb), LOC_OS12G17080 (9.8 Mb), and LOC_OS12G17830 (10.2 Mb) and LOC_OS12G18690 (10.8 Mb), LOC_OS12G19290 (11.2 Mb), and LOC_OS12G20260 (11.8 Mb) (http://rice.plantbiology.msu.edu/). For 11 resistant cultivars carrying *Pi-ta* (Tadukan, Tetep, Te Qing, Yashiro-mochi, Pi4, Reiho, IR64, Katy, Banks, Drew, and Madison), fragments from six additional flanking loci were sequenced: LOC_OS12G12370 (6.8 Mb), LOC_OS12G13570 (7.6 Mb), LOC_OS12G14330 (8.2 Mb), LOC_OS12G22360 (12.6 Mb), LOC_OS12G24020 (13.7 Mb), and LOC_OS12G25630 (14.8 Mb) (http://rice.plantbiology.msu.edu/) (Figure 1).

Sequence data analysis: All DNA sequences from *Pi-ta* and 12 flanking genes were aligned using Vector NTI 10 (Invitrogen) and MEGA 4 (Tamura *et al.* 2007). The genomic sequence from Nipponbare, a *temperate japonica* cultivar, was included as the reference sequence (http://rice.plantbiology.msu.edu/). Additional sequences of the *Pi-ta* gene of 50 accessions of *O. rufipogon*, 3 accessions of *O. nivara*, 2 accessions of *O. meridionalis*, 6 accessions of *O. glaberrima*, and 6 accessions of *O. barthii* were obtained from the GenBank database (Table S1), yielding a total of 226 accessions. For the sequence analysis, accessions of *temperate japonica*, *tropical japonica*, and aromatics collectively formed the *japonica* subspecies, and *aus* and *indica* together formed the *indica* subspecies. Nucleotide polymorphisms at and around the *Pi-ta* region were analyzed using the software DnaSP 4.9 (Rozas *et al.* 2003). The level of nucleotide diversity at silent sites (π_{silent}) and the population mutation parameter θ_w (Watterson estimator) of *Pi-ta* and the flanking gene fragments were estimated for each group of *O. sativa* and compared with that of other Oryza species. Average rates of nonsynonymous (K_a) and synonymous (K_s) substitutions were calculated to examine selections at the *Pi-ta*

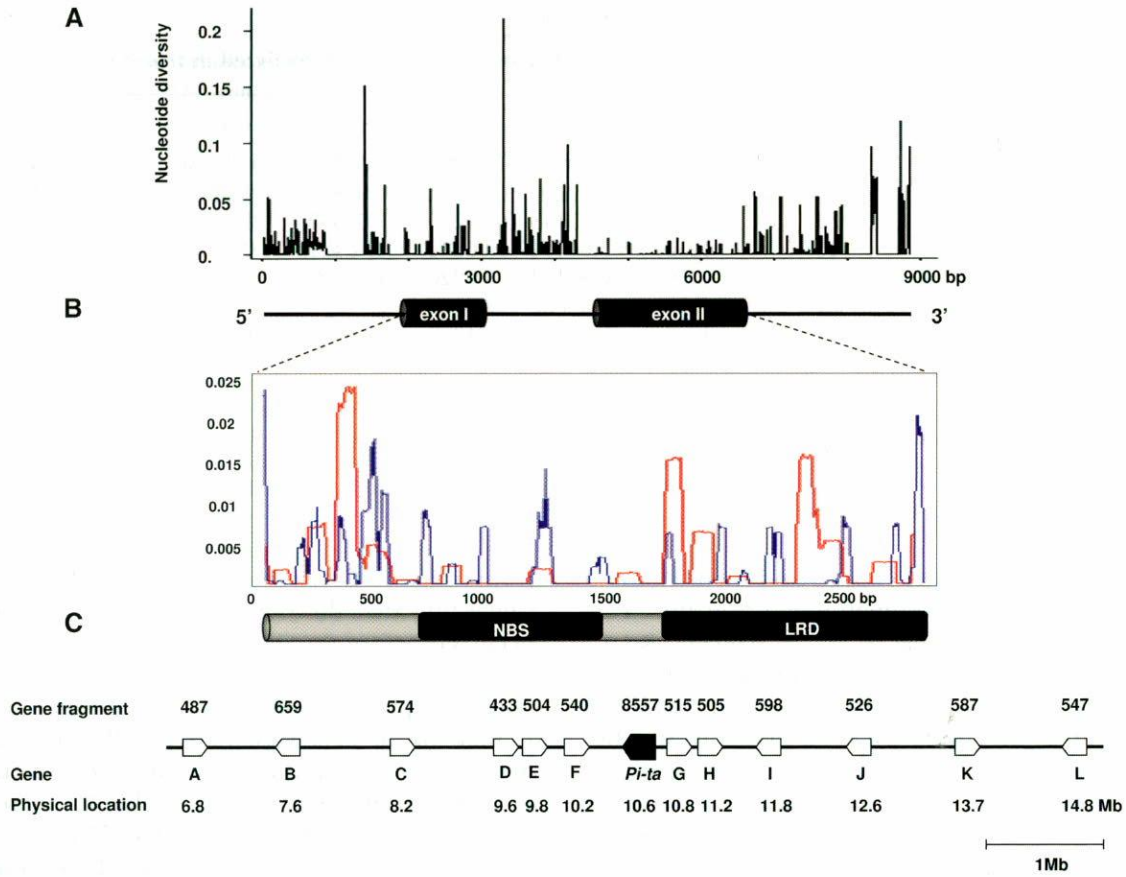


FIGURE 1.—Patterns of DNA sequence variation at and around the *Pi-ta* locus in the AA genome of Oryza species. (A) Sliding-window analysis of the *Pi-ta* locus in 159 accessions (top). The gene structure of *Pi-ta* is shown at the bottom. (B) Sliding-window analysis at the *Pi-ta* coding region (top). The structure of the *Pi-ta* coding region is shown at the bottom. Values were assigned to the nucleotide at the midpoint of 5 bp for A and 25 bp for B, respectively. The parameter of difference per site (*y*-axis) is plotted against the nucleotide position (*x*-axis). Each line indicates synonymous (red) or nonsynonymous variation (blue). (C) Graphic presentation of the genomic region of the *Pi-ta* locus and 12 flanking loci. Sequenced fragments and physical locations on the chromosome are indicated, and the names of loci are represented as A–L. A: outer envelope protein (LOC_OS12G12370); B: Myb-like protein (LOC_OS12G13570); C: NBS–LRR disease resistance protein (LOC_OS12G14330); D: ubiquitin–protein ligase/zinc ion-binding protein (LOC_OS12G16690); E: pentatricopeptide repeat-containing protein (LOC_OS12G17080); F: unknown (LOC_OS12G17830); G: unknown (LOC_OS12G18690); H: serine/threonine-protein kinase (LOC_OS12G19290); I: unknown (LOC_OS12G20260); J: unknown (LOC_OS12G22360); K: senescence-associated protein DIN1 (LOC_OS12G24020); L: sulfite oxidase (LOC_OS12G25630).

coding region in all accessions of *O. sativa* and *O. rufipogon*. Joint analyses of interspecific comparisons using *O. barthii* as an outgroup species were used for estimating the ratio of K_a/K_s and for determining deviations from neutral evolution (AKASHI 1999). Sliding-window analysis was performed to examine nucleotide polymorphism across the *Pi-ta* gene in all Oryza species. Statistical tests of neutrality such as Tajima's *D*, Fu and Li's *D** and *F**, and Fay and Wu's normalized *H* were calculated to examine the selection present at and around *Pi-ta*. Extended haplotype homozygosity (EHH) (SABETI *et al.* 2002) was calculated to visualize the effect of selection on the alleles containing Ala-918 or Ser-918. A haplotype network was also constructed for comparisons of genealogical relationships among *Pi-ta* haplotypes using TCS 1.21 (CLEMENT *et al.* 2000).

RESULTS

Nucleotide diversity at the *Pi-ta* region: High levels of nucleotide variation were observed in the intron, 5′-UTR, and 3′-UTR regions of *Pi-ta* in 159 accessions (Figure 1A). Insertions and deletions (indels) ranging from 10 to 540 bp in the noncoding regions were distinguished among the *Pi-ta* haplotypes. A 242-bp deletion in an intron of *Pi-ta* was found only in *O. glaberrima*, *O. barthii*, and *O. glumaepatula*. Within the coding region, levels of nucleotide and amino acid polymorphism were substantially higher in the first exon. Comparisons of amino acid mutations among partitions of the coding region showed that nonsynonymous were more common than synonymous changes in the NBS region (Figure 1B). Nucleotide diversity in *O. sativa* was lower than that in *O. rufipogon*. A total of 175 polymorphic sites, excluding indels, were found in the coding region, including an intron; of these polymorphic sites, 29 occurred in *O. sativa*, 121 in *O. rufipogon*, and 25 in other Oryza species. Average

TABLE 1

Molecular evolutionary parameters of the *Pi-ta* gene in Oryza species analyzed in this study

Species	Sample no.	Nucleotide	θ_w	π_{silent}	*D*	*D**	*F**	H_n
O. sativa	55	4250	0.00206	0.00287	1.36790	0.43956	0.92805	0.16426
O. sativa indica	23	4250	0.00235	0.00257	−0.02930	−0.18210	−0.15867	−0.29793
O. sativa japonica	32	4250	0.00143	0.00230	1.63577	1.25707	1.62162	0.41988
O. sativa japonica Asian cultivar	16	4250	0.00174	0.00244	0.78421	0.47668	0.64840	0.01420
O. sativa japonica U. S. cultivar	16	4250	0.00174	0.00226	1.41409	1.53348**	1.72883*	0.64640
O. rufipogon	91	3988	0.00888	0.00522	−2.14289*	−2.09795	−2.54113*	−3.65945
O. nivara	5	4003	0.01520	0.01322	−1.06420	−1.06420	−1.15583	−3.39370
O. glaberrima	10	4002	0.00966	0.01366	1.88503	1.03161	1.41069	0.19870
O. barthii	9	4002	0.01066	0.01336	1.21069	1.07971	1.24886	−1.63819

θ_w, Watterson's nucleotide diversity estimator (1975) based on silent site; π, Nei's nucleotide diversity (1987) based on silent site; *D*, Tajima's *D* statistics (1989) based on the differences between the number of segregating sites and the average number of nucleotide differences; *D** and *F**, the neutral test proposed by Fu and Li (1993); and H_n, normalized Fay and Wu's H test statistics. Statistical significance: $^{**}P < 0.02$ and $^{*}P < 0.05$.

pairwise nucleotide diversity (π_{silent}) and silent Watterson's nucleotide diversity estimator (θ_w) over the *Pi-ta* gene was lowest in *O. sativa* (π_{silent} = 0.00292, θ_w = 0.00180) compared to other Oryza species, including *O. rufipogon* (π_{silent} = 0.00522–0.01366, θ_w = 0.00888–0.01520) (Table 1). The levels of diversity in African cultivated rice *O. glaberrima* and its wild progenitor *O. barthii* were similar to *O. rufipogon* and *O. nivara* (Table 1). Analyses for *O. glumaepatula* and *O. meridionalis* were not included because of sample limitation.

A total of 53 *Pi-ta* haplotypes were identified (Table S3) from seven AA genome Oryza species, including the previously reported 20 haplotypes (Huang *et al.* 2008; Wang *et al.* 2008; Yoshida and Miyashita 2009). Among them, 32 *Pi-ta* haplogroups were identified from different Oryza species in the haplotype network, suggesting that the diversification of *Pi-ta* haplotypes occurred before the divergence of these Oryza species (Figure 2). Nineteen haplotypes were from *O. sativa* and 25 haplotypes were from *O. rufipogon* (Table S3). A total of 26 Pi-ta variants from PT1 to PT26 were identified on the basis of the amino acid sequence of the Pi-ta protein in Oryza species (Table 2); these include 8 Pi-ta variants previously identified (Wang *et al.* 2008). Five Pi-ta variants—PT1, PT2, PT3, PT4, and PT20—were the most prevalent type of the variants in *O. sativa* (Figure 2 and Table S1). PT1 containing the functional amino acid alanine at 918 was found only in accessions of *O. sativa* and *O. rufipogon*. PT22, PT23, PT24, PT25, and PT26, were the major types of Pi-ta variants found in

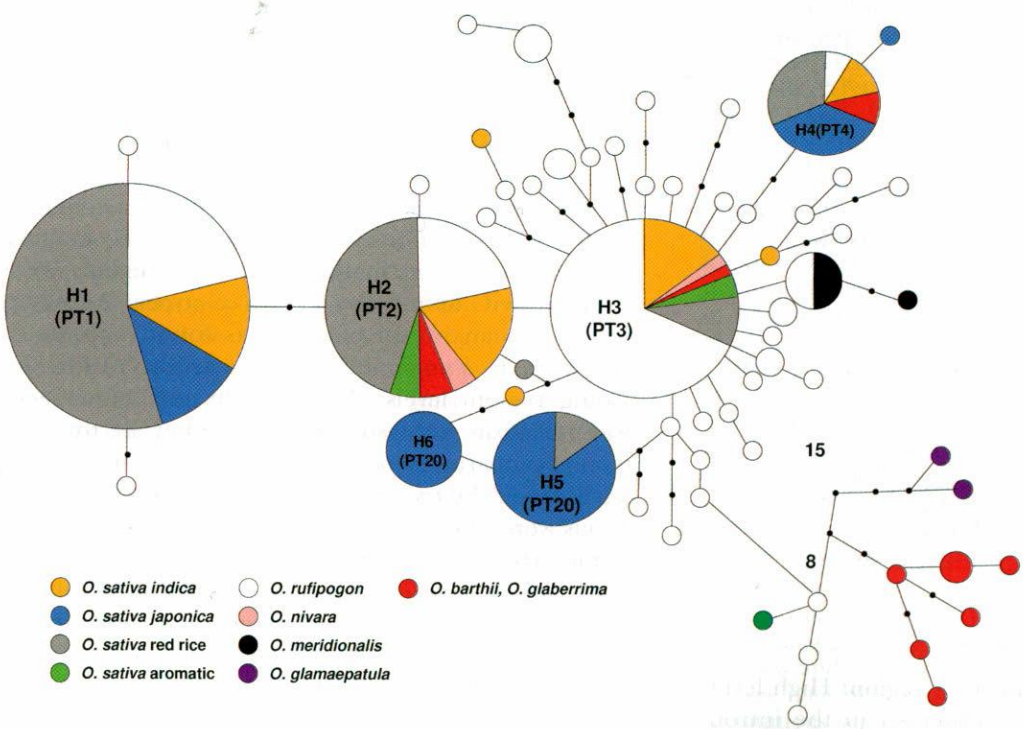


Figure 2.—A haplotype network based on nucleotide polymorphisms of the *Pi-ta* coding region of 226 accessions of seven AA genome Oryza species: *O. sativa*, *O. rufipogon*, *O. nivara*, *O. meridionallis*, *O. glaberrima*, *O. barthii*, and *O. glumaepatula*. Each group of haplotypes is shown as a solid circle, and seven major haplotypes are marked in larger circles. The Pi-ta variants are in parentheses. Each branch represents a single mutational step. Branches with small solid circles indicate that there is more than a single mutational step between haplotypes. A number next to a branch represents the length of the mutational steps. Different sizes of circles represent the different numbers of each haplotype.

TABLE 2

Description of Pi-ta variants based on a 928-amino-acid sequence in 159 accessions of seven Oryza species: *O. sativa*, *O. rufipogon*, *O. nivara*, *O. meridionalis*, *O. glumaepatula*, *O. barthii*, and *O. glaberrima*

Pi-ta variant	Amino acid position: 6 16 30 61 68 78 79 87 102 114 118 131 148 158 162 164 167 174 176 216 230 234 270 315 386 395 403 466 477 479 571 636 644 672 676 711 724 796 810 816 887 891 911 918	Dis.[a]	*Oryza* species
PT1	ILPYPTARTSGLRHHGTEDQKIPRVHMHIKADLLATRRDLHFPA	R	*O. sativa and O. rufipogon*
PT2	I.....S	S	*O. sativa, O. rufipogon, O. nivara, O. glaberrima*
PT3	S.....S	R	*O. sativa, O. rufipogon, O. nivara, O. meridionales, O. glaberrima, O. barthii*
PT4	S.....S.....S	S	
PT5	S...L.....S	S	
PT6	S.....A.....S	-	
PT7	S.....F.....S	S	
PT8	S.....I..E.....S	-	
PT9	S.....V.....S	S	
PT10	S.....G.....S	-	
PT11	SV.....G.....S	-	
PT12	S.....M.....S	-	
PT13	S.....V.S	-	
PT14	S.....G.....N....S	R	
PT15	S.....G.....V.....N.....S	-	
PT16	S.....L.G.....V.....N.....S	-	
PT17	S.....L.G.....S	S	
PT18	S.....V.....S	S	
PT19	S.....V.....N.....S	-	
PT20	S.....SQ....V.....S	S	
PT21	R.....Q....V.....S	-	
PT22	S.....V.....D.....RV.KFYIR.RS.I..NC..FN.LS	S	*O. rufipogon, O. nivara, O. glaberrima, O. barthii, O. glumapatula*
PT23	S..H..V.....D..Q...V.K.YI...S.I..NC..FN..S	S	
PT24	S..H..V.....D.....V.K.YI...S.I..NC..FN..S	S	
PT25	S.....V.....D.....V.K.YI.....I..N...FN..S	S	
PT26	S.....VH.....D.....V.K.YI.....I..N...FN..S	S	

[a] The disease reactions for Pi-ta variants PT1, PT2, PT3, PT4, PT9, PT20, PT22, PT23, PT24, PT25, and PT26 were obtained from two U. S. races, IB17 and IB49 of *M. oryzae* (Wang *et al.* 2008 and this study). Disease reactions for PT5, PT7, PT14, PT17, and PT18 were marked according to the previous report of Huang *et al.* (2008). R, resistance; S, susceptibility.

O. glaberrima, *O. barthii*, and *O. glumaepatula* (Table 1). The EHH test in *O. sativa* revealed that the level of regional recombination around the *Pi-ta* allele (Ala-918) was lower (EHH = 0.331) than that in the allele (Ser-918) (EHH = 0.669). This result suggests that the alanine-918 allele was recently derived from the ancestral Pi-ta variants that carry serine at 918 (Figure 3).

Selection at the *Pi-ta* locus: Tests of neutrality were performed for the *Pi-ta* gene (coding region and intron) using the statistics of Tajima's D, Fu and Li's D^* and F^*, and Fay and Wu's H_n (Table 1). The value of Tajima's D was positive and deviated from neutrality in *O. sativa* ($D = 1.32357$); however, other values for Fu and Li's D^* and F^* and for Fay and Wu's H_n ($D^* = 0.21562$, $F^* = 0.77317$, Fay and Wu's $H_n = 0.16426$) were not significantly different from the neutral model. To determine if the statistical differences were from the population structure of the *Pi-ta* gene in *O. sativa*, accessions separated into four subpopulations—*indica*, *japonica*, *japonica* Asian cultivars, and *japonica* U. S. cultivars—were analyzed for neutral tests. As shown in Table 1, all statistical values for neutral tests did not significantly deviate from neutrality except in U. S. cultivars ($D = 1.41409$, $D^* = 1.53348$, and $I^* = 1.72883$, $P < 0.05$), consistent with the fact that *Pi-ta* has been substantially selected for preventing blast disease in the United States. On the contrary, significant negative values of neutrality tests in *O. rufipogon* suggest an excess of rare alleles, consistent with a recent selective sweep (Table 1) and with the report using different accessions of *O. rufipogon* (Huang *et al.* 2008). Positive values of Tajima's D and Fu and Li's D^* and F^* were found in *O. glaberrima* and its wild ancestor *O. barthii*, suggesting a balancing selection (Table 1). However, it was not

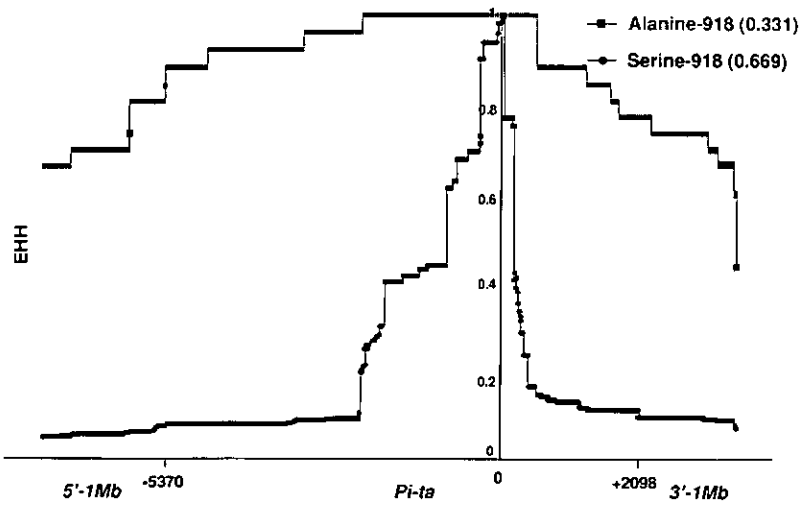


FIGURE 3.—Comparison of the EHH of two core haplotypes (alanine-918 and serine-918) in the *Pi-ta* region in *O. sativa*. The core was defined by a single amino acid change at the position of 918 (serine: TCT or alanine: GCT) that determines the resistance specificity of *Pi-ta*. The starting EHH value for alanine-918 is 0.331 while the EHH value is 0.669 for serine-918.

determined if it was due to selection or population structure in both species because of sample limitation.

The level of synonymous divergence (K_s) exceeded that of nonsynonymous divergence (K_a) in all partitions of the coding region of the *Pi-ta* protein except the NBS region in *O. sativa* and *O. rufipogon*, indicating purifying selection against amino acid substitutions in most portions of the gene (Table 3). These findings were also confirmed in comparisons between synonymous nucleotide polymorphism (π_{syn}) and nonsynonymous nucleotide polymorphism (π_{non}) in *O. rufipogon* (Table 3). However, the π_{syn}:π_{non} ratio was smaller than one (π_{syn}:$\pi_{non} < 1$) in the NBS in *O. sativa* due to the very low polymorphism present in the species. The NBS of the Pi-ta protein in both *O. sativa* and *O. rufipogon* showed a greater number of interspecies nonsynonymous-to-synonymous substitutions ($K_a/K_s > 1$), indicating that positive directional selection has favored amino acid substitutions in this domain (Table 3).

Nucleotide polymorphisms in genomic regions around *Pi-ta*: We sequenced all fragments of targeted flanking loci around *Pi-ta* except one locus encoding a NBS–LRR disease resistance protein (LOC_OS12G14330), the RPM-1 homolog located at 8.2 Mb. The presence and absence of the RPM-1 homolog was found in both *O. sativa* and *O. rufipogon* accessions. The absence of the RPM-1 homolog was found in two Asian cultivars, Yashiro-mochi (*japonica*) and Te Qing (*indica*), and in all U. S. weedy rice carrying resistant *Pi-ta* (Table S4).

Nucleotide data sets shown in Figure 1 were aligned for 433–659 bp of six loci in 2 Mb around *Pi-ta* in all 159 accessions. The estimated values of nucleotide diversity for these loci were 0–0.00391 in *O. sativa* and 0.0015–0.00508 in *O. rufipogon*. The levels of sequence variation in flanking loci around *Pi-ta* were similar to the levels in the *Pi-ta* locus found in both species (Table 4). The test of Tajima's *D* in the region around *Pi-ta* in *O. sativa* and *O. rufipogon* revealed that no significant pattern of

TABLE 3

Molecular variation and selection at the *Pi-ta* gene in *O. sativa* (*indica*, *japonica*, and weedy rice) and *O. rufipogon*

Gene segment	S	π^a_{syn}	π^a_{non}	π_{non}/π_{syn}	K_s(JC)[b]	K_a(JC)[b]	K_a/K_s[b]
			O. sativa ($n = 113$)				
Coding	12	0.0015	0.00098	0.654	0.00871	0.00560	0.643
5′ coding to NBS	6	0.00305	0.00337	1.105	0.01513	0.01034	0.683
NBS	2	0.00009	0.00003	0.295	0.00004	0.00451	102.5
NBS to LRD	0	0	0	0	0.03457	0.00371	0.107
LRD	4	0.00196	0.00063	0.319	0.00774	0.00536	0.692
			O. rufipogon ($n = 91$)				
Coding	62	0.00249	0.00166	0.668	0.00849	0.00530	0.625
5′ coding to NBS	24	0.00491	0.00278	0.564	0.01211	0.00698	0.577
NBS	15	0.00101	0.00155	1.543	0.00052	0.00484	9.341
NBS to LRD	4	0.00401	0.00049	0.121	0.03384	0.00359	0.106
LRD	19	0.00174	0.00139	0.801	0.00694	0.00514	0.741

[a] π_{syn}, nucleotide diversity at synonymous site; π_{non}, nucleotide diversity at nonsynonymous site.

[b] Jukes–Cantor (JC) corrected synonymous differences per synonymous site (K_s) and nonsynonymous differences per nonsynonymous site (K_a) using intraspecific and interspecific comparisons using *O. barthii*.

TABLE 4

Molecular diversity of genomic regions around *Pi-ta* in *O. sativa* (*indica*, *japonica*, and weedy rice) and *O. rufipogon*

	Nucleotide polymorphism (π)						Tajima's *D*					
Physical location (Mb)	9.6	9.8	10.2	*Pi-ta*[a]	10.8	11.2	11.8	9.6	9.8	10.2	*Pi-ta*[a]	10.8	11.2	11.8
O. sativa	0.00178	0.00115	0.00108	0.0018	0.0015	0	0.00391	0.66938	0.69958	0.82595	**0.17149**	0.92414	NA	0.91739
O. indica	0.0019	0.00109	0.00204	0.00218	0.00213	0	0.00479	−0.96803	−1.51481	−0.10605	**−0.70826**	1.08052	NA	1.02022
O. japonica	0.00118	0.00148	0.00047	0.00253	0.00048	0	0.00416	0.97327	0.27501	0.44003	**1.05235**	0.6426	NA	0.13462
Weedy rice	0.001	0.00129	0.0004	0.00166	0.00168	0	0.00365	−0.93379	−0.45271	−0.51132	**−0.44628**	−0.42536	NA	1.13812
O. rufipogon	0.00508	0.0015	0.0057	0.00477	0.00301	0.0015	0.00388	−0.15872	0.23998	0.16801	**−2.57275**	0.22133	−1.36029	−0.3066

[a] The sequence of *Pi-ta* including flanking region (2 kb upstream and downstream of *Pi-ta*) and coding region with intron was used for nucleotide polymorphism and Tajima's *D*.

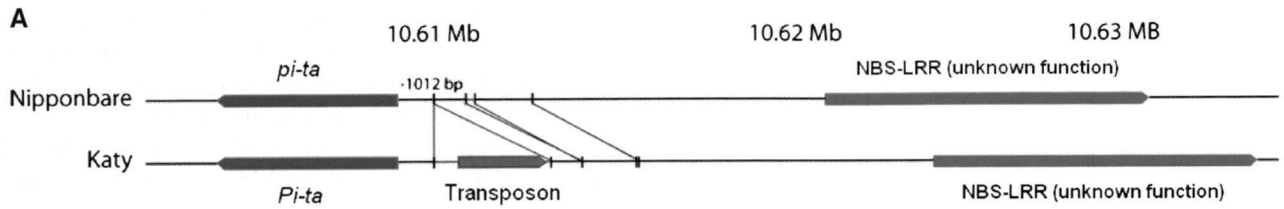
selection presents around the *Pi-ta* locus in *O. sativa*. However, a significant negative value of Tajima's *D* was detected around the *Pi-ta* locus in *O. rufipogon*, similar to the result found in the *Pi-ta* gene (Table 4).

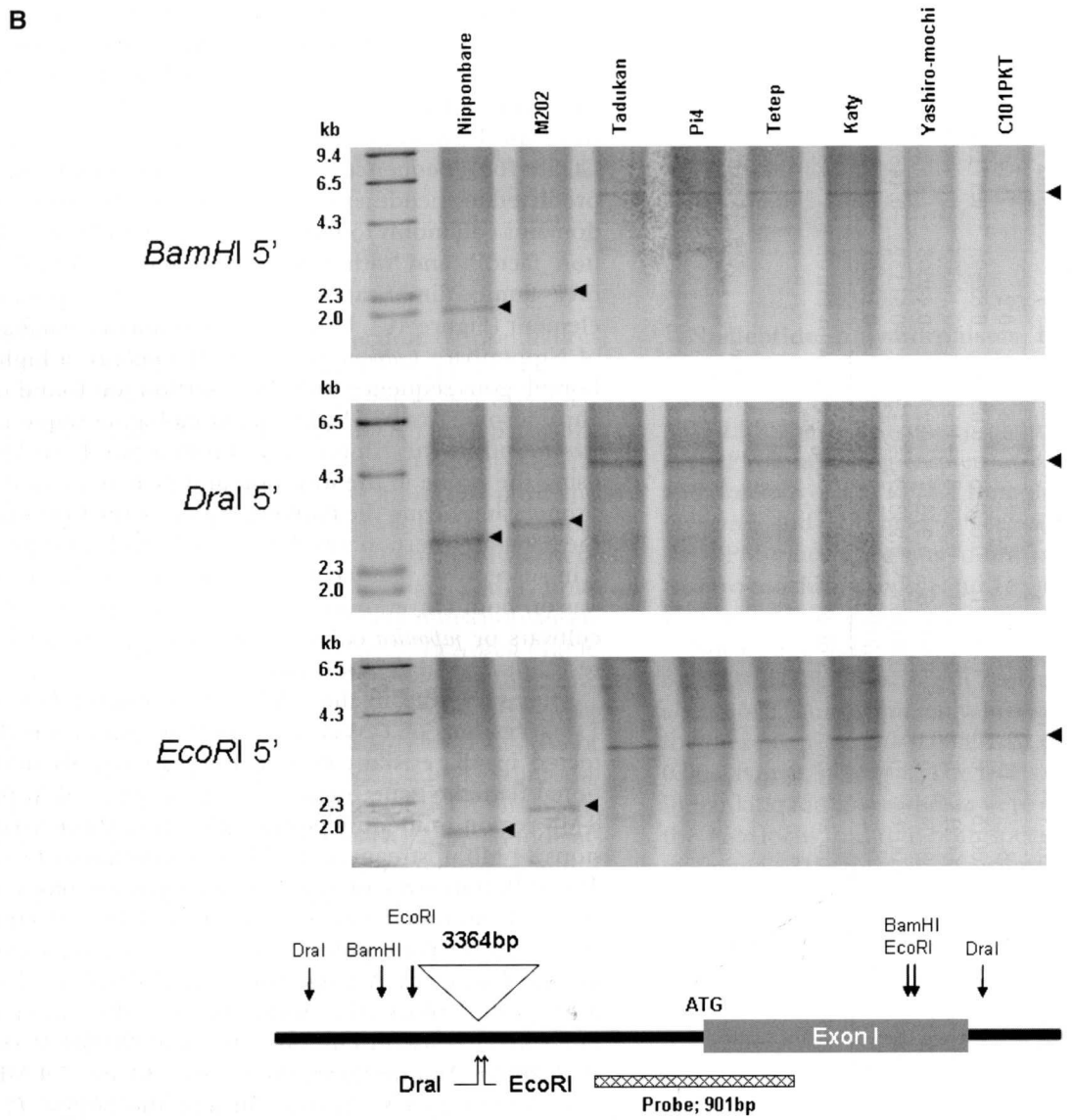
Interestingly, a 3364-bp insertion located 1012 bp upstream of the start codon (ATG) was found only in all accessions carrying the resistance *Pi-ta* allele (Figure 4A). The presence of the insertion in resistant accessions was verified by Southern blot analysis using a probe derived from the 5′ region of *Pi-ta* (Figure 4B). The inserted fragment was cloned and sequenced from the U. S. cultivar, Katy (GenBank accession no. GQ984160). Sequences of the 3364-bp fragment were predicted to encode a protein with 844 amino acids with domains commonly found in zinc fingers and transcription factors and with domains commonly found in hAT family dimerization (hATC) of a transposable element (Figure 4C). Using the rice sequence database of Nipponbare (*japonica*) and 93-11 (*indica*), a highly homologous sequence with the insertion was found on chromosome 2 of 93-11 while no homologous sequence was found in the Nipponbare. From a Southern blot using the probe in the insertion and PCR analysis with primers amplifying the flanking region of the insertion, the 3364-bp insertion was determined on chromosome 2 in susceptible *indica* cultivars; however, the insertion was on both chromosomes 2 and 12 in resistant *indica* cultivars or *japonica* cultivars possessing *indica*-derived resistant *Pi-ta* (data not shown).

After surveying in the 2-Mb region around *Pi-ta* in 118 accessions of *O. sativa*, no polymorphism was detected in all resistant *O. sativa* accessions. Six additional flanking gene fragments were sequenced in the 8-Mb region to identify polymorphisms in those accessions (4 Mb upstream and 4 Mb downstream of *Pi-ta*). The different sizes of the *Pi-ta* introgressed block in resistant cultivated rice were estimated by detecting the initial breaking point of recombination surrounding the *Pi-ta* locus. A range from 5 to 8 Mb of the *Pi-ta* introgression block (the average being 7 Mb) was identified in 11 resistant cultivars (Jia *et al.* 2004b; Wang *et al.* 2007). Among them, the smallest block (5.4 Mb) was identified in Yashiro-mochi and the largest *Pi-ta* introgression (>8 Mb) was found in the two Japanese cultivars Pi4 and Reiho whose *Pi-ta* region was derived from Tadukan. A 6.8-Mb portion of the *Pi-ta* region in Tetep was identified in the U. S. cultivars Katy, Drew, Banks, and Madison (Figure 5).

DISCUSSION

In this study, we analyzed DNA sequence polymorphisms in and around the genomic region of *Pi-ta* in 159 geographically diverse Oryza accessions composed of several Oryza species to gain insight into the origin and evolution of *Pi-ta*. We discovered that the extended genomic region (>5 Mb) surrounding resistant *Pi-ta*





C

```
MSSRNRKYDSGAEKRKKRKRLEAVAQSQKGALDKFFLRETPNANIEDDISDDMAEVDANIAESDDAVEENVDGDIGHDLADEGRDLASEGNEENIADDAD  [100]
                                                                                ZnF_TTF
DNVSFRPDMFDPRTWDGLDPKMIDILLQKGPKRDLSIEHGPRDNLSRRFLASSYTKVLSNGEKCDREWLVYSKELDKVFCFCCKLLRKGLVRGQLANDGV  [200]
NDWNHLANRLKEHEVSREHVTNMSTWYELRLRMQKNQTIDKVAQRELEKEREHWRRVLLRILLIVKFLAEHNIAFRGSNSKLYQDSNGNFLGLVEMLVEF  [300]
DPVIKEHVDRITNDKIRDHYLGPSIQNELINLLAVAIKSSIIAKIKEAKYFSVILDCTPDASHQEQMSLIIRYVDVTTCSIEESFLGFLDVNDTSGQGLF  [400]
DVLVEELNSLDLDVANVRGQGYDNGSNMKGKHQGVQKKLLDINPRAFYSACGCHSLNLTLCDMAKSCRKATEFFGVIQRIYTTFANSTKRWKILKDNLSG  [500]
LTLKSLSSTRWESRVDSVKAIRFQIPEIREALLQVAETDNDPLTVSEVNSLSENELGGFEFLVAIIIWYEILSSINVVSKQLQSKDMVIDIAIESVQGLI  [600]
SLFKKYRENGFSKALEAAKQIALEMDIPIEFRTKRKIKRKRQFDEGTSDASIDSQSGEESFRINYFIPVVDQAIASLIRRFEQYQGYEKTFGFLFTSDRL  [700]
                                                                                hATC
RLLDDDSLLAACENLEVALKSGEHKDIDGKELSDELGLIQQILKKSMGPLDILQFLKERPFYPNATVAYRILLTIPVTVASAERSFSKLKLLKSYLRSTM  [800]
TQERLNGLATIALEKDILEKINYEDIIEDFISRNTRRMMLFSTS* [845]
```

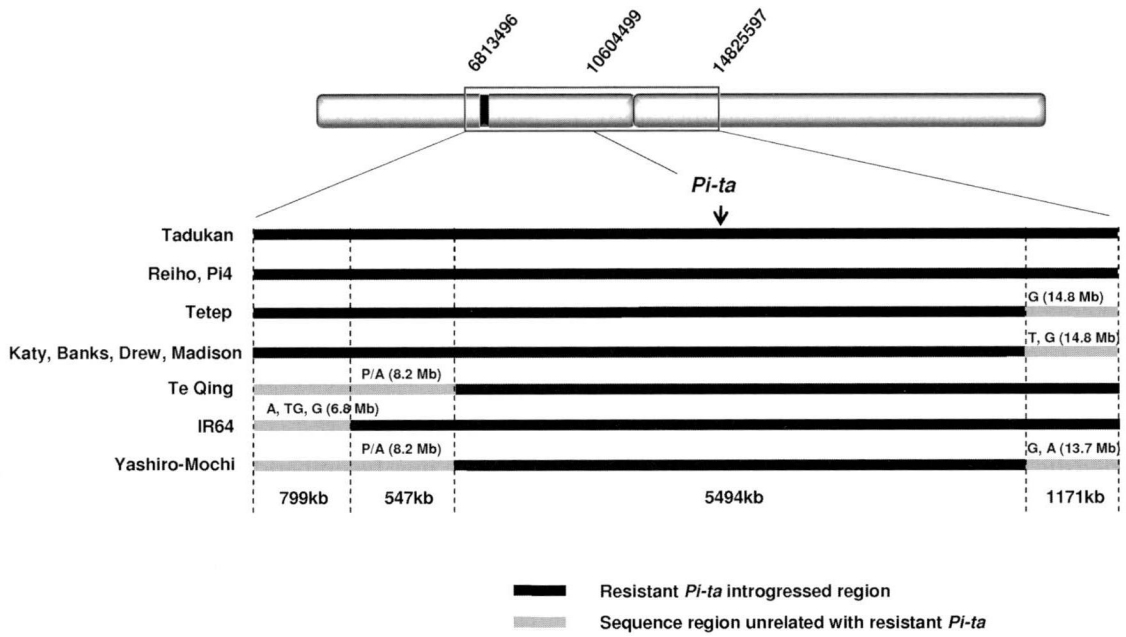


FIGURE 5.—Sizes of *Pi-ta* introgressions in *O. sativa* Asian and U. S. cultivated rice through breeding selection during domestication. The *Pi-ta* region of Tadukan, which is the major donor for *Pi-ta* in Asian cultivars, was used to compare the size of the introgression block with other *Pi-ta*-containing cultivars. The solid bar represents the identical sequence of *Pi-ta* introgressed into resistant cultivars. The shaded bar represents sequence polymorphisms unrelated to the *Pi-ta* introgression that resulted from recombination events at the genomic region of *Pi-ta*. Sequence polymorphisms are marked on the breakpoint of the *Pi-ta* introgression block. P/A indicates the presence and absence of polymorphism.

was consistently maintained in resistant accessions to *M. oryzae* containing *AVR-Pita*. Significantly, one of the largest linkage blocks of resistant *Pi-ta* was identified in backcrossing and elite rice cultivars (JIA 2009). The identification of a large linkage block around *Pi-ta* raised at least two possibilities. First, other blast *R* genes in the *Pi-ta* region also introgressed into diverse elite rice cultivars. Other *R* genes such as *Pi-ta*^{2}, *Pi39*, and *Pi20(t)* (RYBKA *et al.* 1997; LIU *et al.* 2007; LI *et al.* 2008) were also mapped at the *Pi-ta* region, but it was unknown if these and/or other unknown *R* genes were clustered in the *Pi-ta* region that have been introgressed as a large linkage block. Second, other components for the *Pi-ta*-mediated resistance reside within the 5-Mb region to form a superlocus. *R*-gene-mediated resistance may involve additional *R* genes that may be physically linked to provide a complete resistance to a plant pathogen. In tomato, Prf, a NBS–LRR protein, was identified to be involved in the Pto-mediated resistance (MUCYNA *et al.* 2006). In rice, at least two NBS–LRR proteins at the *Pikm* and *Pi5* loci have been identified as providing complete resistance to blast (ASHIKAWA *et al.* 2008; LEE *et al.* 2009). At the *Pikm* locus, *Pikm1-TS* and *Pikm2-TS* within 2.5 kb are required for *Pikm*-mediated disease resistance (ASHIKAWA *et al.* 2008). Similarly, two NBS–LRR proteins within 50 kb, *Pi5-1* and *Pi5-2*, were required for complete resistance (LEE *et al.* 2009). At the *Pi-ta* locus, another gene *Ptr(t)* was found to be essential for *Pi-ta*-mediated resistance (JIA and MARTIN 2008). The possible artificial selection of the large *Pi-ta* genomic region has been reported for maintaining the broad spectrum of *Pi-ta*-mediated blast resistance (JIA 2009). Taken together with other studies, this study suggests that other components such as *Ptr(t)* or *R* genes for the *Pi-ta*-mediated resistance may occur within at least 5 Mb of the *Pi-ta* region.

Simple insertion/deletion or transposon may play an important role in *R*-gene evolution. It has been reported that 18.8% of total *R* genes in Arabidopsis and 22.2% in rice are under presence/absence polymorphism (MEYERS *et al.* 2003; SHEN *et al.* 2006). An example of transposon and *R*-gene activation was found in the *Pit* gene. The insertion of a long-terminal-repeat retrotransposon in the promoter of *Pit* was predicted to regulate *Pit* transcription and its function for resistance (HAYASHI and YOSHIDA 2009). In our study, we found a transposon

FIGURE 4.—Genomic organization around *indica* (resistant *Pi-ta*) and *japonica* (susceptible *Pi-ta*) cultivars. (A) Comparisons of genomic regions around the *Pi-ta* locus between Nipponbare and Katy. (B) An insertion in the proximate *Pi-ta* promoter region differentiates the size of hybridized bands between two susceptible cultivars (Nipponbare and M202) and six resistant cultivars (*Pi-ta*) (top). Schematic of the *Pi-ta* genomic region with indicated restriction enzymes (bottom). (C) The two domains (shaded)—zinc finger in transposases and transcription factors (ZnF_TTF) and hAT family dimerization (hATC)—were identified by searching the conserved domain of proteins from NCBI database.

in the proximity of the *Pi-ta* promoter in resistant cultivars carrying *Pi-ta*, which was absent in accessions without *Pi-ta*. This finding suggests that the transposon may activate the *Pi-ta*-mediated resistance. Further study may lead to a better understanding of any associations of the transposon with *Pi-ta*-mediated resistance.

The divergence of *indica* and *japonica* subgroups in *O. sativa* was predicted to be caused by two independent domestications from geographically divergent *O. rufipogon* populations (Londo and Schaal 2007). The *Pi-ta* haplotypes of *indica* or *japonica* origin were identified in this study (Figure 2). Resistant *Pi-ta* was found only in *indica*, weedy rice, *japonica* cultivars carrying the *indica*-derived *Pi-ta* region and *O. rufipogon*, suggesting that resistant *Pi-ta* did not originate from *japonica*. The Pi-ta variants in H5 and H6 were found only in *japonica* accessions, while H2 and H3 were found only in *indica* (Figure 2), consistent with a previous study (Londo and Schaal 2007). The Pi-ta variant containing Ala-918 (PT1) separates the resistant Pi-ta variant from other variants in both *O. sativa* and *O. rufipogon*. This suggests that PT1 existed before the divergence of the two subspecies *indica* and *japonica*. The recent divergence of resistant *Pi-ta* from susceptible *Pi-ta* has also been proposed from the previous studies (Huang *et al.* 2008; Yoshida and Miyashita 2009). Most of the Pi-ta variants possess serine at the position of 918. There was no amino acid sequence polymorphism in the group with PT1; however, significant amino acid polymorphism was identified in groups containing Ser-918, consistent with previous reports (Huang *et al.* 2008; Wang *et al.* 2008; Yoshida and Miyashita 2009). These findings further suggest that there was recently a strong selection constraint on the resistant Pi-ta protein (PT1), and such pressures were not observed on other Pi-ta protein variants.

An excess of amino acid substitutions over neutral expectations were observed in the NBS region in both *O. sativa* and *O. rufipogon*, indicating that positive directional selection favored amino acid substitutions in the domain. The NBS domain in diverse proteins with ATP or GTP binding activity is involved in activating the NBS–LRR protein in resistance. It has been documented that the Toll-interleukin 1 receptor region of the *L* class of flax rust *R* genes (Ellis *et al.* 1999) and the N-terminal domain with the NBS region of tomato MI protein (Hwang *et al.* 2000) are key regulators of signal transduction of disease resistance. Our findings suggest that the highly diversified NBS region may be important for maintaining the integrity of the Pi-ta protein with the LRD domain. In the LRD of the Pi-ta protein, the level of synonymous diversity was found to exceed the level of nonsynonymous diversity, which is suggestive of possible purifying selection acting on this domain. The K_a:K_s ratio for the LRD of Pi-ta (K_a:K_s = 0.692–0.741) is relatively low compared to that observed in other LRRs (Ellis *et al.* 1999; Mauricio *et al.* 2003; Rose *et al.* 2004; Bakker *et al.* 2006; Orgil *et al.* 2007). It is possible that conservation of LRD in the Pi-ta protein may be necessary for recognizing AVR-Pita for the signal transduction (Jia *et al.* 2000). High nucleotide diversity and a large number of *AVR-Pita* haplotypes were recently identified, suggesting that *AVR-Pita* is under diversifying selection (Y. Dai and Y. Jia, unpublished data). Diversified selection at NBS and purifying selection against amino acid variants in the conserved functional LRD region may have played a major role in shaping the molecular evolution of *Pi-ta*.

In conclusion, this study revealed that (1) a transposon may be a part of the evolution with resistant *Pi-ta*, (2) all components needed for the *Pi-ta*-mediated resistance may be embedded within 5 Mb, and (3) strong artificial selection has acted at and around resistant *Pi-ta* in the modern cultivated rice *O. sativa*, while such selection is absent in cultivars without resistant *Pi-ta*. These findings suggest that the evolution of *Pi-ta* is much more complicated than previously documented. Further studies will be necessary for a better understanding of the molecular mechanism of *Pi-ta*-mediated signal recognition and transduction pathway.

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